IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT INTERFERENCES AND APPEALS

Application No.	:	10/540,956	Confirmation No. :	5119
Applicants	:	Kumar Tyagi et al.		
Filed	:	March 13, 2006		
Title	:	NOVEL GENE OSISAP1 OF RICE CONFERS TOLERANCE TO STRESSES AND A METHOD THEREOF		
Group Art Unit	:	1638		
Examiner	:	Vinod Kumar		
Customer No.	:	28289		

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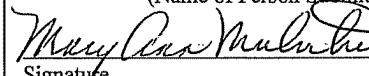
APPLICANT'S APPEAL BRIEF

Sir or Madam:

On June 17, 2008, Applicants filed a Notice of Appeal, thereby appealing the rejections asserted in the Final Office Action dated December 17, 2007, and the Advisory Action dated March 17, 2008. Applicants now submit this Appeal Brief, and respectfully request that the asserted rejections be reversed, and that the presently pending claims be found novel and patentable over the prior art, and in condition for allowance.

I hereby certify that this correspondence is being electronically submitted to the United States Patent and Trademark Office on August 15, 2008.

Mary Ann Mulvihill
(Name of Person Submitting Paper)



Signature 08/15/2008
Date

REAL PARTY IN INTEREST

The University of Delhi, having it principal place of business located at Benito Juarez Road, New Delhi, India 110 021, who is the assignee and present owner of this patent application (see Reel 017300 Frame 0374), is the real party in interest.

RELATED APPEALS AND INTERFERENCES

The undersigned, as the representative for the University of Delhi and counsel for this Application, has no knowledge of appeals or interferences that relate to this application or appeal.

STATUS OF CLAIMS

Claims 1-34 have been cancelled. Claims 35-45 are presently pending, and stand rejected. Applicants hereby appeal the rejection of claims 35-45.

STATUS OF AMENDMENTS

After the mailing of the Final Office Action, Applicants filed an Amendment dated February 15, 2008, which contained amendments to the claims. In particular, claims 44 and 45 were amended. According to the Advisory Action of March 17, 2008, these amendments were entered. Thus, Applicants believe that all amendments have been entered.

SUMMARY OF CLAIMED SUBJECT MATTER

In this appeal, Applicants argue that independent claim 35 is patentable over the cited prior art, and for the same reasons, claims 36-45, which depend from claim 35, are likewise patentable.

Claim 35 recites a method of increasing abiotic stress tolerance in a plant (for example, see Specification at page 3, lines 11-12; and page 4, lines 12-31). The method comprises transforming the plant with a recombinant vector (for example, see Specification at page 3, lines 19-21; page 4, lines 4-6 and 18-29). The recombinant vector comprises a polynucleotide sequence set forth in SEQ ID NO: 1 or a variant thereof (for example, see Specification at page 3, lines 16-18; and page 4, lines 1-3 and 18-29). The polynucleotide sequence codes for a polypeptide sequence as shown in SEQ ID NO: 2 (for example, see

Specification at page 3, lines 16-18; page 4, lines 18-29). The polynucleotide sequence or the variant thereof is expressed to yield transformed plants (for example, see Specification at page 3, lines 22-24).

Since none of the dependent claims are separately argued, a summary of the dependent claims is not provided pursuant to the waiver of this requirement under 37 C.F.R. § 41.37 (c)(1)(v).

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 35-45 have been rejected under 35 U.S.C. § 103(a) as unpatentable over Mukhopadyay *et al.* (NCBU/EMBL Database, Seq. Access. No. AF140722, Publ. June 7, 2000) (hereinafter "Mukhopadyay") in view of Hiei *et al.* ("Efficient transformation of rice (*Oryza sativa L.*) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA," THE PLANT JOURNAL, (1994) 6(2): 271-282) (hereinafter "Hiei") and Liu *et al.* ("Transcription factors and their genes in higher plants," EUR. J. BIOCHEM., (1999) 262: 247-257) (hereinafter "Liu"). Applicants respectfully request that this rejection be reviewed on appeal and reversed.

ARGUMENT

This rejection should be reversed because it fails to establish a *prima facie* case of obviousness. Particularly, the requisite reason to use a cytoplasmic protein rather than a transcription factor taught by the references, and/or a AN1/AN20 type zinc-finger protein has not been established.

As part of a *prima facie* case, an examiner must establish some reason to combine the references. *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 131 (2007); *Takeda Chemical Industries, Ltd. v. Alpharpharm Pty., Ltd.*, 492 F.3d 1350, 1356-1357 (Fed. Cir. 2007). The *KSR Int'l* Court acknowledged the importance of identifying a reason that would have prompted a person of ordinary skill in the art to combine the elements in the way the claimed invention does. *KSR Int'l*, 127 S.Ct. at 1731; *Takeda Chemical*, 492 F.3d at 1356-1357. Repeatedly throughout the *KSR Int'l.* decision, the Court discussed the importance that the result obtained by a particular combination was predictable to one of ordinary skill in the art. *KSR Int'l*, 127 S.Ct. at 1731 and 1739-1742.

A combination of known elements will not yield predictable results if the references disclose a broad selection of compounds or combinations, or if the references

teach away from the claimed invention. See *Takeda Chemical*, 492 F.3d at 1359; also see *Ortho-McNeil Pharmaceutical, Inc. v. Mylan*, 520 F.3d 1358, 1364 (Fed. Cir. 2008); and also see *Ex parte Ikeda*, App. No. 08/352,079, Appeal 2008-0492, Slip Op. at 7 (BPAI Mar 26, 2008). For example, in *Takeda Chemical*, the post-*KSR* Federal Circuit noted that the recited compound, which was a modified version of compound b, was not recognized at the pertinent time as a suitable candidate for treatment of Type II diabetes. 492 F.3d at 1359. *Takeda Chemical* involved United States Patent No. 4687,777, which was directed to a compound for the treatment of Type II diabetes. *Id.* 1352-1354. The defendant argued that the patent was obvious in view of a reference that disclosed compound b, because the claimed compound could be synthesized from compound b by routine means. *Id.* at 1357. However, the Federal Circuit affirmed that the patent was not obvious because the defendant had not explained the reason why one would have selected compound b in the first place. *Id.* at 1360.

The Federal Circuit further held that the prior art, in fact, taught away from choosing compound b as a starting point. *Id.* at 1359-1361. Compound b was known to have unwanted side effects, and there was nothing in the prior art to suggest that homologation would decrease the unwanted side effects. *Id.* at 1359-1360.

In a more recent case, the Board reversed an examiner's rejection for failing to provide the requisite reason to combine the references. *Ikeda*, App No. 08/352,079 at 7. The *Ikeda* application was directed to a method of removing hydrocarbons from exhaust gases. *Id.* at 2. In pertinent part, the claims recited an absorption catalyst B located downstream of a catalyst A in the direction of the exhaust gas. The claims were rejected as unpatentable under 35 U.S.C. § 103 in view of Swaroop, Abe and Patil. *Id.* at 3. Swaroop taught positioning the absorption catalyst B upstream catalyst A. *Id.* at 5. To remedy the deficiency in the art, the examiner cited "Patil and Abe as evidence of the 'conventionality of positioning the adsorbent catalyst 1 either upstream or downstream of a [three-way] catalyst 3' and thus conclude[d] that it would have been obvious to one of ordinary skill in this art to select an appropriate location for the adsorbent catalyst 16 in the apparatus of Swaroop ...." *Id.* at 5-6. The Board held that

> The Examiner has failed to provide any cogent reason or technical discussion to support the conclusion that one of ordinary skill in this art would have employed the relative positions of the catalysts in Abe and Patil without the use of the other teachings of these references, namely an auxiliary heater and

> bypass lines with valving. Second, the Examiner has not explained why one of ordinary skill in this art would have used the teachings of Patil, requiring bypass lines and valving, when Swaroop specifically teaches away from the use of valving and bypass lines [*citation omitted*]. Third, the Examiner has not supplied convincing reasoning or technical discussion to support the proposed switch in relative position of the catalysts when Swaroop specifically teaches that the exhaust gas is "modified" by the adsorbent catalyst and this modified form of the exhaust gas is *then* sent to the main or three-way catalyst to undergo conversion to innocuous products [*citation omitted*]. ... Fourth, the Examiner has not explained why one of ordinary skill in this art would have proceeded contrary to the teachings of Patil, namely the teachings that "it is not possible merely to place zeolite 'in-line' in the exhaust system with the [main] catalyst has reached an effective temperature and unconverted hydrocarbons would still be discharged to the atmosphere" [*citation omitted*].

Id. at 7.

Like *Takeda Chemical* and *Ikeda*, the Examiner here has also failed to meet this burden. The Examiner has failed to establish why a person of ordinary skill in the art would use a gene that is a cytoplasmic protein in view of references that disclose using only transcription factors. Additionally, there is no reason such a person could predict that an AN1/AN20 type zinc-finger protein would be involved in stress tolerance when no reference has every identified any of these types of zinc-fingers to be involved in stress tolerance.

The invention as recited in claim 35 is directed to a method of increasing abiotic stress tolerance in a plant. The method comprises transforming the plant with a recombinant vector. The recombinant vector comprises a polynucleotide sequence for *OsiSAP1*. As evidenced by page 15 of the Kanneganti *et al.* article, *OsiSAP1* is a cytoplasmic protein, not a transcription factor. The article specifically states that:

> Generally many of the zinc finger domain proteins act as transcriptional factors. However, it was hypothesized that *OsiSAP1*, may not act as transcriptional factor since it lacks any nuclear localization signal and DNA binding domain. It was also suggested that *OsiSAP1* might carry out its function via protein-protein interactions (Mukhopadhyay *et al.* 2004).

By way of background, plants are made of many eukaryotic cells. A eukaryotic cell is a cell that comprises a nucleus. The nucleus contains the cell's DNA, which is enclosed in a nuclear envelope. Transcribing a DNA to an RNA occurs in the nucleus by an enzyme called an RNA polymerase. This process is directly regulated by transcription factors. Transcription factors are proteins that binds to the DNA at a specific

site via a DNA binding domain positioned within the transcription factors. By binding to the DNA, the transcription factor either increases or inhibits the ability of the RNA polymerase to transcribe the DNA.

In order for a transcription factor to work, it must be able to enter the nucleus. This is accomplished by a nuclear localization signal. A nuclear localization signal is an amino acid sequence that permits the protein to enter the nucleus. Proteins that have nuclear localization signals can enter the nucleus via the nuclear pore.

The area outside the nucleus is called the cytoplasm. Hence, cytoplasmic proteins are proteins that reside in the cytoplasm, not the nucleus, and do not contain nuclear localization signals. Consequently, cytoplasmic proteins have no direct contact with DNA.

Mukhopadhyay discloses a DNA sequence from *Oryza sativa*, which the Examiner contends discloses the sequence for the recited *OsiSAP1*.

Hiei is directed to plant transcription factors. Unlike the *OsiSAP1* gene, these transcription factors contain nuclear location signals and a DNA-bind region, among other functional segments. In pertinent part, Hiei notes the importance of transcription factors with regard to gene expression. It states that the

> Alteration in the expression of transcription factor genes normally results in dramatic changes to a plant [*citations omitted*] and structural changes to these genes may represent a significant evolutionary force [*citation omitted*]. As a practical consequence, ***engineering of transcription factor genes provides a valuable means for manipulation of plants*** [*citation omitted*], but success in such endeavors depends on how well the genes are understood.

(Emphasis added, Hiei at p. 247). The references make no mention of cytoplasmic proteins, such as *OsiSAP1*, or their role in manipulation of a plant's gene expression.

The Examiner contends, in conclusory fashion, that it would be obvious to use a cytoplasmic protein, as recited in the claims, instead of a transcription factor without explaining the requisite reason to do so. Ignoring the evidence that the recited gene is a cytoplasmic protein and not a transcription factor, the Examiner provides the following:

> In the instant case, it was well known in the art that zinc finger motif(s) containing proteins are transcription factors which are implicated in a plant's

> response to abiotic stresses. Thus, it would have been obvious and within the scope of an [sic] ordinary skill in the art to combine the teachings of Mukopadhyay et al., Hiei et al., and Liu et al. to arrive at the instantly claimed invention with reasonable expectation of success.

(Office Action at page 5). In the Examiner's only effort to address the fact that *OsiSAP1* is a cytoplasmic protein, the Examiner contends that "it would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to transform a plant with Mukhopadhyay et al. polynucleotide sequence encoding a zinc finger motif(s) containing protein using any method of plant transformation including the rice plant transformation method taught by Hiei et al. ...." (Advisory Action at page 2.)

However, in view of Hiei, which specifically states that "***engineering of transcription factor genes provides a valuable means for manipulation of plants***," the Examiner has still failed to establish why one of ordinary skill in the art would use a cytoplasmic protein. The Examiner's obviousness argument is limited to: it is obvious to use Hiei's method to transform a plant with any gene. What is lacking is any reason to use this specific gene – that one of ordinary skill in the art could predict that *OsiSAP1*, a cytoplasmic protein, would increase abiotic stress tolerance in plants.

This case is similar to *Takeda Chemical* because the examiner in *Takeda Chemical* failed to establish a reason to use compound b in the first place. 492 F.3d at 1360. Likewise, the Examiner here has failed to establish a reason to use the cytoplasmic protein transcribed from the *OsiSAP1* gene. Based on Hiei, one of ordinary skill in the art would at best expect that transcription factors may be implicated in a plant's response to abiotic stresses. *OsiSAP1* is **not** a transcription factor because it lacks a nuclear localization sequence and a DNA binding domain, and there is no reason provided to use genes that encode cytoplasmic proteins.

This case is also similar to *Ikeda* because the examiner in *Ikeda* failed to explain why one would ignore Swaroop's teaching of placing the absorption catalyst B upstream of catalyst A. Appeal No. 2008-0492 at 5-7. Likewise, the Examiner here has also failed to explain why one would ignore Hiei's and Liu's teachings that certain transcription factors implicate abiotic stress tolerance, and instead look at wholely unrelated cytoplasmic proteins such as *OsiSAP1*.

Additionally, on page 6, the Office Action contends that, in view of Liu, plant transcription factors with zinc-finger motif(s) are implicated in abiotic stress tolerance in a plant. Again, according to Kanneganti *et al.*, *OsiSAP1* is not a transcription factor. Therefore, even assuming that Liu can be extended to teach that all plant transcription factors with zinc finger motif(s) are implicated in abiotic stress tolerance in a plant, Liu, nevertheless, does not teach that cytoplasmic proteins (which are not transcription factors) having zinc-finger motif(s) are implicated in a plant's stress tolerance.

According to Kanneganti *et al.*, one of ordinary skill in the art would not recognize Mukhopadhyay's gene sequence to code for a transcription factor since it lacks a nuclear localization signal or a DNA binding domain. Since the Examiner has not ptovided a single reason why one of ordinary skill in the art would expect that a cytoplasmic protein to increase abiotic stress tolerance in plants, a *prima facie* case of obviousness has not been established.

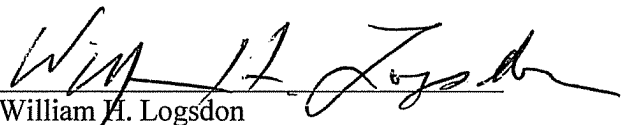
In addition, Liu does not teach or suggest that an AN1/A20 type zinc-finger gene would be involved in stress tolerance. Not all zinc-finger proteins are transcription factors, nor are all zinc-finger genes related to stress tolerance. In fact, the claimed invention is the first AN1/A20 type zinc-finger gene linked to stress tolerance. In contrast, Liu describes five classes of zinc-finger transcription factors, none of which is a AN1/A20 type zinc finger protein. Thus, the Examiner has further failed to explain why one of ordinary skill in the art would expect an AN1/A20 type zince finger protein to be linked to stress.

Conclusion

In light of the array of transcription factor genes and other genes that are involved in stress tolerance, the claimed invention is patentable over the cited references because the inventors are the first to identify the specific SAP1 (AN1 and A20 type) zinc-finger gene that is involved in stress tolerance. Accordingly, Applicants respectfully request that the asserted rejections be reversed, and that the claims 35-45 be found patentable over the cited prior art and in condition for allowance.

Respectfully submitted,

THE WEBB LAW FIRM

By 

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CLAIMS APPENDIX

Claims 1-34 (Canceled)

Claim 35 (Previously Presented) A method of increasing abiotic stress tolerance in a plant, said method comprising transforming said plant with a recombinant vector comprising a polynucleotide sequence set forth in SEQ ID NO: 1 or a variant thereof coding for a polypeptide sequence as shown in SEQ ID NO: 2, and expressing the polynucleotide sequence or the variant thereof to yield transformed plants.

Claim 36 (Previously Presented) The method of claim 35, wherein said plant used for transformation is selected from a group consisting of tobacco, rice and tomato plant.

Claim 37 (Previously Presented) The method of claim 35, wherein said method provides transformed plants having increased tolerance to cold stress relative to an untransformed plant of the same plant species.

Claim 38 (Previously Presented) The method of claim 35, wherein said method provides transformed plants having increased tolerance to drought stress relative to an untransformed plant of the same plant species.

Claim 39 (Previously Presented) The method of claim 35, wherein said method provides transformed plants having increased tolerance to salt stress relative to an untransformed plant of the same plant species.

Claim 40 (Previously Presented) A transgenic plant, plant tissue or plant cell produced by the method of claim 35, wherein said transformed plant, plant tissue or plant cell exhibits increased tolerance to cold stress relative to an untransformed plant of the same plant species.

Claim 41 (Previously Presented) A transgenic plant, plant tissue or plant cell produced by the method of claim 35, wherein said transformed plant, plant tissue or plant cell exhibits increased tolerance to drought stress relative to an untransformed plant of the same plant species.

Claim 42 (Previously Presented) A transgenic plant, plant tissue or plant cell produced by the method of claim 35, wherein said transformed plant, plant tissue or plant cell exhibits increased tolerance to salt stress relative to an untransformed plant of the same plant species.

Claim 43 (Previously Presented) Transgenic seeds produced by the transgenic plant of claim 40, wherein the seeds comprise the polynucleotide sequence.

Claim 44 (Previously Presented) Transgenic seeds produced by the transgenic plant of claim 41, wherein the seeds comprise the polynucleotide sequence.

Claim 45 (Previously Presented) Transgenic seeds produced by the transgenic plant of claim 42, wherein the seeds comprise the polynucleotide sequence.

EVIDENCE APPENDIX

1. Kannegant *et al.*, "Overexpression of OsiSAP8, a member of stress associated protein (SAP) gene family of rice confers tolerance to salt, drought and cold stress in transgenic tobacco and rice," PLANT MOL. BIOL. (2008). A copy was previously provided February 15, 2008. A courtesy copy is enclosed with this Appeal Brief.

RELATED PROCEEDINGS APPENDIX

None.

Plant Mol Biol
DOI 10.1007/s11103-007-9284-2

Overexpression of *OsiSAP8*, a member of stress associated protein (SAP) gene family of rice confers tolerance to salt, drought and cold stress in transgenic tobacco and rice

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Abstract We describe here the isolation and characterization of *OsiSAP8*, a member of stress Associated protein (SAP) gene family from rice characterized by the presence of A20 and AN1 type Zinc finger domains. *OsiSAP8* is a multiple stress inducible gene, induced by various stresses, namely heat, cold, salt, desiccation, submergence, wounding, heavy metals as well as stress hormone Abscisic acid. OsiSAP8 protein fused to GFP was localized towards the periphery of the cells in the epidermal cells of infiltrated *Nicotiana benthamiana* leaves. Yeast two hybrid analysis revealed that A20 and AN1 type zinc-finger domains of OsiSAP8 interact with each other. Overexpression of the gene in both transgenic tobacco and rice conferred tolerance to salt, drought and cold stress at seed germination/seedling stage as reflected by percentage of germination and gain in fresh weight after stress recovery. Transgenic rice plants were tolerant to salt and drought during anthesis stage without any yield penalty as compared to unstressed transgenic plants.

Keywords A20 and AN1 Zinc finger domains · GFP-fusion · *Oryza sativa* · *OsiSAP8* · Yeast two hybrid · SAP · Stress tolerance

OsiSAP8 is deposited in the Genbank with the Accession number AY345599.

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Introduction

Abiotic stresses, such as drought, salinity, extreme temperatures, high light intensities, exposure to heavy metals are serious threats to agriculture, as they adversely affect the growth and productivity of crop plants. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crops by more than 50% (Boyer 1982; Bray et al. 2000). Most abiotic stresses directly or indirectly lead to the production of free radicals and reactive oxygen species, creating oxidative stress (Oberschall et al. 2000). The burden of environmental stress on crop plants is likely to increase because of the climate changes associated with global warming. Furthermore, with extension of crop cultivation to environments which are not optimal for the growth of crop plants, development of stress tolerant plants is becoming increasingly important (Kathuria et al. 2007). Transgenic approaches offer new opportunities to improve tolerance to abiotic stresses. Present engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance conferring proteins (reviewed by Wang et al. 2003; Vinocur and Altman 2005; Valliyodan and Nguyen 2006; Sreenivasulu et al. 2007; Kathuria et al. 2007). One important way of achieving tolerance to multiple stress conditions is to overexpress transcription factor gene(s) that control multiple genes from various pathways (Kasuga et al. 1999; Oh et al. 2005; Ito et al. 2006; Hsieh et al. 2002; Kim et al. 2004; Lee et al. 2006) or by overexpressing genes involved in abiotic signal perception and transduction (Shou et al. 2004; Teige et al. 2004; Saijo et al. 2000).

In the recent past, a new family of genes termed as SAP (Stress Associated protein) gene family was studied in rice for its role in abiotic stress conditions by expression profiling under those conditions (Vij and Tyagi 2006). SAP gene family members are characterized by the presence of A20/AN1 domain in their putative encoded proteins. Abstract proteins with A20/AN1 zinc-finger domain are present in all eukaryotes and are well characterized in animals. From *Oryza sativa* and *Arabidopsis* genome, 18 and 14 genes, respectively, coding for SAP related proteins (SAP family) were identified (Vij and Tyagi 2006). The majority was found to have both the A20 zinc-finger domain (present at the N-terminus) and the AN1 zinc-finger domain (present at the C-terminus). This is consistent with the previous finding from animal systems that the A20 and AN1 zinc-finger domains are usually found associated with each other (Evans et al. 2004).

Although the role of some of the A20/AN1 zinc-finger proteins in regulating the immune response has been well worked out in animal systems (Hishiya et al. 2006), very little information is available on such proteins from plants. The *indica* rice *Osi*SAP1 (*Oryza sativa indica* stress Associated protein 1) was identified as the first plant protein having A20 and AN1 zinc-fingers present at the N- and C-terminus, respectively. It is found to be an intron-less gene present as a single copy gene in rice genome. *Osi*SAP1 transcript was detected at higher levels in root and pre-pollination stage panicle. It was also shown to be overexpressing under several abiotic stresses like cold, salt, desiccation, submergence, heavy metals, mechanical wounding as well as with stress hormone abscisic acid (ABA) with in 15 min of treatment, hence the product might be required early after stress. In addition, overexpression of this gene in transgenic tobacco lead to an increased stress tolerance as determined by cold-, dehydration- and salt-tolerance assays. Overexpression of *Osi*SAP1 could help avoid stress-associated injuries such as chlorosis and cell death in transgenic plants and better recovery from stress (Mukhopadhyay et al. 2004).

The mechanism or mode by which *Osi*SAP1 confers stress tolerance is not known. From the nature of early induction, the *Osi*SAP1 gene product might act early in the signal transduction pathway of stress response (Mukhopadhyay et al. 2004). It may use its zinc-finger domains for protein–protein interactions, as shown in case of ZnF216 protein in humans with similar domain architecture (Scott et al. 1998). Co-immuno precipitation studies have shown that the A20 domain of ZnF216 interacts with itself, AN1 domain and protein kinase domain of other proteins. AN1 domain did not interact with itself but found to interact with A20 domain (Huang et al. 2004).

Interestingly, all the members of the rice SAP gene family present in the rice genome showed inducibility to one or the other abiotic stresses (Vij and Tyagi 2006). It would be important to define the relative function of the members of this gene family in the life of the rice plant. Here in this study, we describe the isolation and functional characterization of *OsiSAP8*, a member of SAP gene family in rice which is induced by various abiotic stress treatments like salt, drought, cold, heat, desiccation, submergence, wounding, heavy metals and ABA. Sub-cellular localization of OsiSAP8-GFP fusion protein indicated it to be a cytoplasmic protein. Yeast two hybrid analysis indicated that A20 and AN1 zinc-finger domains of OsiSAP8 interact with each other. Thus, the present work provides the evidence for the first time that SAP gene family members may be cytoplasmic proteins and might carry out their functions via protein–protein interactions aided by A20 and AN1 zinc-finger domains. Overexpression of *OsiSAP8* in transgenic rice and tobacco plants conferred tolerance to high salt (800 mM NaCl), drought (Water deficit for 23 days during anthesis) and cold (4 ± 1°C for 4 days) stresses.

Materials and methods

Plant materials and treatments

Indica rice cv *IR-50 and Nicotiana benthamiana* were used in the present study. Healthy seeds of rice were surface sterilized with 70% ethanol, rinsed thoroughly, soaked in double distilled water overnight, and the seedlings were raised on the water saturated cotton in a Biotron PH100 growth chamber (Nippon, Japan) maintained at 28°C, 80% relative humidity and 16 h photoperiod (180 $\mu Em^{-1}\ s^{-1}$). After 8 days of growth, they are transferred to 100 ml beakers containing water (mock solution) and kept at 4 ± 1°C for cold stress and 42°C for heat stress. The seedlings were also treated with 250 mM NaCl for salinity stress, 400 mM mannitol for drought stress. Desiccation stress was simulated by drying the seedlings on tissue paper and keeping them wrapped in dry tissue paper for the desired time. For submergence stress, the beaker with seedlings was submerged under water in a 2 l glass beaker. Wounding stress inflicted by clipping the leaf margins at 1 cm intervals. For treatment with DMSO, cut leaves of seedlings were kept at 25°C for 6 h in presence of 2 and 4% DMSO and control was kept at 25°C for 6 h in water. Abscisic acid (ABA) was dissolved in DMSO to make a stock of 10 mM and was diluted further in water. The seedlings were also treated with 50 mM of various sulphates or chlorides of Zinc, Cadmium, Mercury, Lithium, Calcium, Manganese and Magnesium for heavy metal stress.

Isolation of *OsiSAP8*

In this study, an attempt was made to isolate cDNA inserts from rice root cDNA library (cloned in Lambda ZAP vector, stratagene) by PCR amplification. For this, a small number of random clones from rice root cDNA library were picked up and the cDNA inserts cloned directionally in the *Eco*RI (5′ end) and *Xho*I (3′ end) sites of phagemid vector were PCR amplified using T3 and T7 primers specific for the T3 and T7 promoter regions located on either side of multiple cloning site (MCS). About ~1.2 kb amplicon obtained from one of the clone (phage lysate) was electro-eluted and cloned into *Xcm*I digested pXcmKn12 vector (National Institute of Genetics, Japan) to obtain pKVZn. The total cDNA sequence of 1,132 bp was obtained by end sequencing of cDNA clone using M13F and M13R primers. The sequence was deposited in the Genbank with the accession number AY345599. Bioinformatic analysis revealed it to be identical to *Os*SAP8 from *japonica*, hence the gene was designated as *OsiSAP8* (*Oryza sativa indica* Stress Associated Protein 8).

RNA blot analysis

Total RNA was isolated using TRIreagent kit (Sigma Aldrich, Bangalore, India) according to the manufacturer's protocol. RNA gel blot analysis was performed using NorthernMax Kit (Ambion, USA) according to manufacturer's instructions. About 10 μg of total RNA was electrophoresed on a 1% formaldehyde gel and blotted onto a positively charged nylon membrane (BrightStar-Plus Membrane, Ambion, USA). The membrane was probed with α-^{32}P labeled *OsiSAP8* cDNA fragment, using standard procedures (Sambrook and Russell 2001). Hybridization was detected by autoradiography. Ethidium-bromide-stained rRNA bands from identical samples served as control for total RNA quantity and quality.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from the rice seedlings subjected to various stress treatments using TRIreagent kit (Sigma Aldrich, Bangalore, India) according to the manufacturer's protocol. RT-PCR using 3 μg of DNase-treated RNA samples was performed with 1 μM OligodT (15 mer), 1 μM random hexamers, 1× RT buffer, 10 μM dNTPmix (MBI Fermentas), 10 U RNase inhibitor (MBI Fermentas) and 200 U M-MLV reverse transcriptase (MBI Fermentas). The reaction mixture was incubated at 42°C for 1 h and then M-MLV was heat inactivated at 92°C for 2 min. PCR was performed in a volume of 25 μl containing the appropriate amount of cDNA template, 20 μM each of dNTP, 2 mM $MgCl_2$, 5 μM of random primer (decamers), 1× PCR buffer and 1 U Taq DNA Polymerase (MBI Fermentas). The cycle parameters in the PCR programme were as follows: 94°C for 3 min, [25 cycles for GAPDH (housekeeping gene control) and 30 cycles for *OsiSAP8*] of: 94°C for 30 s, 55°C for 30 s, 72°C for 2 min followed by a final extension at 72°C for 7 min. The following primer sets were used to amplify each transcript specifically: (SAP8for: 5′-ATGGAGCACAAGGAGACT-3′, SAP8rev: 5′-CTAAAT TTTGTCAAGTTTCTC-3′; GAPDHfor: 5′-GGCGCAGCA GCTCTCCGC-3′, GAPDHrev: 5′CTCAGCTCCAAAGT TATCC-3′). About 20 μl of each PCR product was analyzed by agarose gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 μg ml^{-1}). To ensure reproducibility the experiment was replicated; both experiments produced identical results.

Construction of GFP-fusion

Total mGFP was amplified from pBINmGFP vector using the primers 5′-CGCGGATCCATGGTGAGCAAGGGC GAGGAG-3′ and 5′-ACGCGTCGACTTACTTGTACAG CTCGTCCATG-3′. *Bam*HI and *Sal*I restriction sites (underlined) were introduced to facilitate cloning in the corresponding sites of pBTEX binary vector to generate pBTEX-GFP. The coding region sequence of *OsiSAP8* without the stop codon, amplified using the primers 5′-ACTGGTACCATGGAGCACAAGGAGACT-3′ (*Kpn*I site is underlined) and 5′-GCCGGATCCAATTTTGT CAAGTTTCTC-3′ (*Bam*HI site is underlined) from the full-length clone pKVZn, was cloned into the *Kpn*I and *Bam*HI sites of pBTEX-GFP vector to generate pBTEX-GFP-SAP8 construct. The constructs pBTEX-GFP and pBTEX-GFP-SAP8 were individually mobilized in *Agrobacterium* strain EHA105 (Hood et al. 1993) using freeze-thaw transformation (Chen et al. 1994).

Plant agro-infiltration and confocal microscopy

Leaves of *N. benthamiana* were infiltrated with *Agrobacterium* strains containing the above constructs (Llave et al. 2000). After 48 h of infection, the leaves were harvested and observed under a laser scanning confocal microscope for detecting the fluorescence. The images were acquired by an Olympus IX81/FV500 confocal microscope using an argon laser (488 nm), a green helium/neon laser (543 nm) and the PLAPO60XW/LSM/NA1.00 WD 0.15 mm objective. Image analysis was carried out using the Fluoview 500 software.

Yeast two hybrid analysis

For analyzing the interactions between A20 and AN1 zinc-finger domains of *OsiSAP8*, first, A20 region and AN1 region were PCR amplified using ZnA20F 5′-GAATTC CAGCCAAAAGGCCCGA-3′ and ZnA20R 5′-CTCGAG GCTCCTGCTTCATTAT-3′; ZnAN1F 5′-GAATTCCCC AAGGGGAGGGAAG-3′ and ZnAN1R 5′-CTCGAGCGA TAGTCAAACTGGC-3′, respectively. Restriction sites (underlines) were included to facilitate cloning into the appropriate vectors pEG202 (LexA DNA binding vector) and pJG4-5 (LexA activation domain vector). The PCR products were first cloned into T-tailed vector (pXcmKn12) followed by cloning into yeast vectors resulting in pJGA20, pEGA20, pJGAN1 and pEGAN1 constructs. EGY48$^+$ (Erica Golemis Yeast), which is deficient in synthesis of histidine, tryptophan and leucine (Ausubel et al. 1995) was used as host cells. Competent cells of *Saccharomyces cerevisiae* strain EGY48$^+$ for transformation were prepared according to the Sigma yeast transformation kit and were co-transformed with the yeast two-hybrid vector constructs pEGAN1, pJGAN1, pEGA20 and pJGA20 and empty vectors pEG202 and pJG-4-5 in different combinations. To eliminate the false positives, empty vector pEG202 was used as a negative control along with pJGAN1 or pJGA20 to transform the EGY48$^+$ cells. Transformants were selected on selective dropout medium with out histidine, tryptophan and uracil. For qualitative analysis of β-galactosidase activity, the colony-lift filter assay on Whatman #3 was used. Quantitatively, β-galactosidase activity was assayed in liquid culture using *o*-nitrophenyl β-D galactoside (ONPG) as a substrate. Both the assays were done according to the yeast protocols in Clontech manual. For quantifying yeast cell growth, cells were grown in dropout minimal medium lacking tryptophan, histidine and uracil to $OD_{600} = 0.5$. Serial dilutions of the cultures were prepared and a 10 µl sample of each dilution was spotted onto minimal medium deficient for leucine, tryptophan, histidine and uracil.

Plant transformation

To overexpress *OsiSAP8* in tobacco (*Nicotiana benthamiana*) and rice (*Oryza sativa* cv IR-50), the cDNA was cloned under Ubiquitin promoter and Nos terminator in pUC19 vector backbone. The resulting vector, pUbSAP8s and another vector pHX4 (harboring hygromycin phosphotransferse (*hph*) gene under CaMV35s and Nos terminator) were co-bombarded into tobacco and rice explants by biolistic gene delivery system. Rice (cv IR-50) tissue culture, bombardment and selection of hygromycin resistant calli were done according to the protocols of Anoop and Gupta (2003). For biolistic transformation of tobacco, leaf discs were used as explants and the procedure was same as used for rice transformation (Anoop and Gupta 2003) with small differences in the culture medium. MS medium with 0.1 mg/l NAA + 1.0 mg/l BAP + 0.25 M sorbitol + 0.25 mannitol + 0.25% phytagel was used as osmoticum and MS medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BAP was used for subculture. The transformed leaf discs were selected on selection medium (MS medium supplemented with 0.1 mg/l NAA, 1.0 mg/l BAP and 50 mg/l hygromycin B). The integration of transgene in different rice and tobacco lines was confirmed by southern analysis, and for RNA blot analysis, RNA was isolated from leaves to evaluate the expression of introduced gene. *OsiSAP8* cDNA was used as radiolabeled probe for both southern and northern blot analysis.

Leaf disc assay

One cm leaf segments were cut from both rice and tobacco and were floated on 0, 200, 400, 600 and 800 mM NaCl solution for 72 h and at the end of 72 h chlorophyll retention in each sample was calculated. Total chlorophyll estimation from rice and tobacco leaves was performed using DMF method (Porra 2002). About 20 mg leaf tissue added to the eppendorf tube containing 800 µl DMF (*N-N* dimethyl Formamide) and incubated in dark for 2 h at room temperature. After completion of 2 h, the OD of the solvent was taken at 663.8 and 646.8 nm wavelengths. The total chlorophyll content was calculated using the following formula: $[\text{Chl } a] = 12.00 \times \text{A663.8} - 3.11 \times \text{A646.8}$; $[\text{Chl}\,b] = 20.78 \times 646.8 - 4.88 \times \text{A663.8}$; $[\text{Chl}\,a + b] = 17.67 \times \text{A646.8} + 7.12 \times \text{A663.8}$.

Analysis of tobacco transgenics for abiotic stress tolerance

To test the ability of the transgenic tobacco to survive in high salt conditions, analysis was performed at germination stage. At the germination stage, surface sterilized control (wild-type) and transgenic seeds were placed in 1/2 strength MS medium with out sucrose supplemented with 150 mM NaCl. After 7 days of incubation at 28°C, %-germination, root and shoot lengths and fresh weights were determined. To test the ability of the transgenic tobacco to survive in low water conditions, analysis was performed at germination stage. At the germination stage, surface sterilized control (wild-type) and transgenic seeds were placed in 1/2 strength MS medium with out sucrose supplemented with 300 and 400 mM mannitol. After 7 days of incubation at 28°C, percentage germination, root

and shoot lengths and fresh weights were determined. Later the seedlings were transferred back to water and grown for 8 more days for recovery. For cold stress analysis, 12-day-old tobacco seedlings grown on 1/2 strength MS medium with out sucrose in sterile petriplates were transferred to 4 ± 1°C for 96 h. At the end of 4 days, the plate with seedlings was transferred back to 28°C for 15 days for recovery. At the end of the recovery period, the fresh weights of the seedlings were measured. PCR analysis with *OsiSAP8* specific primers indicated that all the transgenic lines are salt tolerant and all salt sensitive lines are non-transgenic. Thus, the contribution of salt sensitive (segregating, non-transgenic) seedlings in T_1 generation that did not harbor the transgene was excluded from the total number of seedlings analyzed for each line to assess the stress tolerance. All experiments were repeated at least twice and the average values were represented.

Analysis of rice transgenics for abiotic stress tolerance

For salt stress analysis at germination stage, transgenic rice seeds were surface sterilized and placed in petriplates having cotton beds saturated with 200 mM NaCl solution. Seeds were allowed to germinate for a period of 7 days under 16 h L/8 h D period at 28°C. At the end of the 7 days, the germinated seeds were counted and the percentage germination was calculated. Root and shoot lengths along with fresh weight were also determined. After 7 days, the plantlets were transferred to water and grown hydroponically for 8 more days for recovery and at the end of recovery phase again the root and shoot lengths along with fresh weight were determined. For stress at plantlet stage, 30-day-old rice plants grown in the pots were subjected to salt stress with 200 mM NaCl for 4 weeks and transferred to another pot without salt and allowed to grow and set seeds. The number of spikes and number of mature seeds obtained per each plant were counted. To test the ability of the transgenic rice plants to survive in low water conditions, analysis was performed at germination stage and anthesis stage. At the germination stage, surface sterilized control (wild-type) and transgenic seeds were placed in 300 and 400 mM mannitol solution saturated cotton beds in sterile petriplates. After 7 days of incubation at 28°C, % germination, root and shoot lengths and fresh weights were determined. Later the plantlets were transferred to water and grown hydroponically for 7 days for recovery. After recovery phase, the shoot and root lengths and fresh weight of the seedlings were measured. Drought stress at the anthesis stage was given by with drawing water from 120-day-old mature plants for 23 days, and the agronomic traits like plant height, panicle number and number of seeds set were also determined. For cold stress analysis, 12-day-old rice seedlings grown hydroponically were transferred to 4 ± 1°C for 96 h. At the end of 4 days, the seedlings were transferred to water at 28°C for 5 days for recovery. At the end of the recovery period, the fresh weights were measured.

Computer analysis

We performed the BLAST search of the rice genomic database at NCBI (http://www.ncbi.nlm.nih.gov/Blast/Genome/PlantBlast.shtml, Altschul et al. 1997) for the genomic sequence of this gene. Various tools from Expasy (http://www.expasy.org/tools) were used to deduce the translated product and compute theoretical pI and molecular weight. The putative domains were identified using the interproscan search (http://www.ebi.ac.uk/interproscan/, Quevillon et al. 2005). The degree of aminoacid sequence identity was determined by the use of Wu-Blast from EBI (http://www.ebi.ac.uk/blast2, Altschul et al. 1990). Multiple sequence alignments involved use of ClustalW (http://www.ebi.ac.uk/clustalW, Thompson et al. 1994) The *cis*-acting regulatory elements in the promoter region were predicted using PLACE database (http://www.dna.affrc.go.jp/PLACE, Higo et al. 1999).

Results and discussion

OsiSAP8 is a single copy gene and belongs to SAP gene family from rice

Computational translation of the 1,132 bp cDNA clone isolated from rice root library indicated that its 516 bp coding region codes for a protein of 171 aminoacids with a predicted molecular mass of 18.4 kDa (Fig. 1A). Homology searches run with the full-length aminoacid sequences of the cDNA clone revealed significant similarity to Stress Associated proteins from Rice and *Arabidopsis* (Vij and Tyagi 2006). The identity values at the aminoacid levels varied in between 99 to 40% with the *Oryza sativa* SAPs and 61 to 36% with that of *Arabidopsis* SAPs. Since the cDNA clone has shown 99% identity to *OsSAP8* from *japonica* rice, it was designated as *OsiSAP8* (*Oryza sativa indica* stress associated protein 8). In silico analysis revealed that its actual transcript size is of 1,239 bp with a coding region of 516 and 151 bp of 5′ untranslated region and 559 bp of 3′ untranslated region.

The conserved domain search of OsiSAP8 aminoacid sequence in NCBI and the interproscan in EBI server predicted the presence of two Zinc finger domains characteristic of SAP gene family in plants, namely ZnF-A20 at N-terminus end, spanning from 11–45 aa residues and

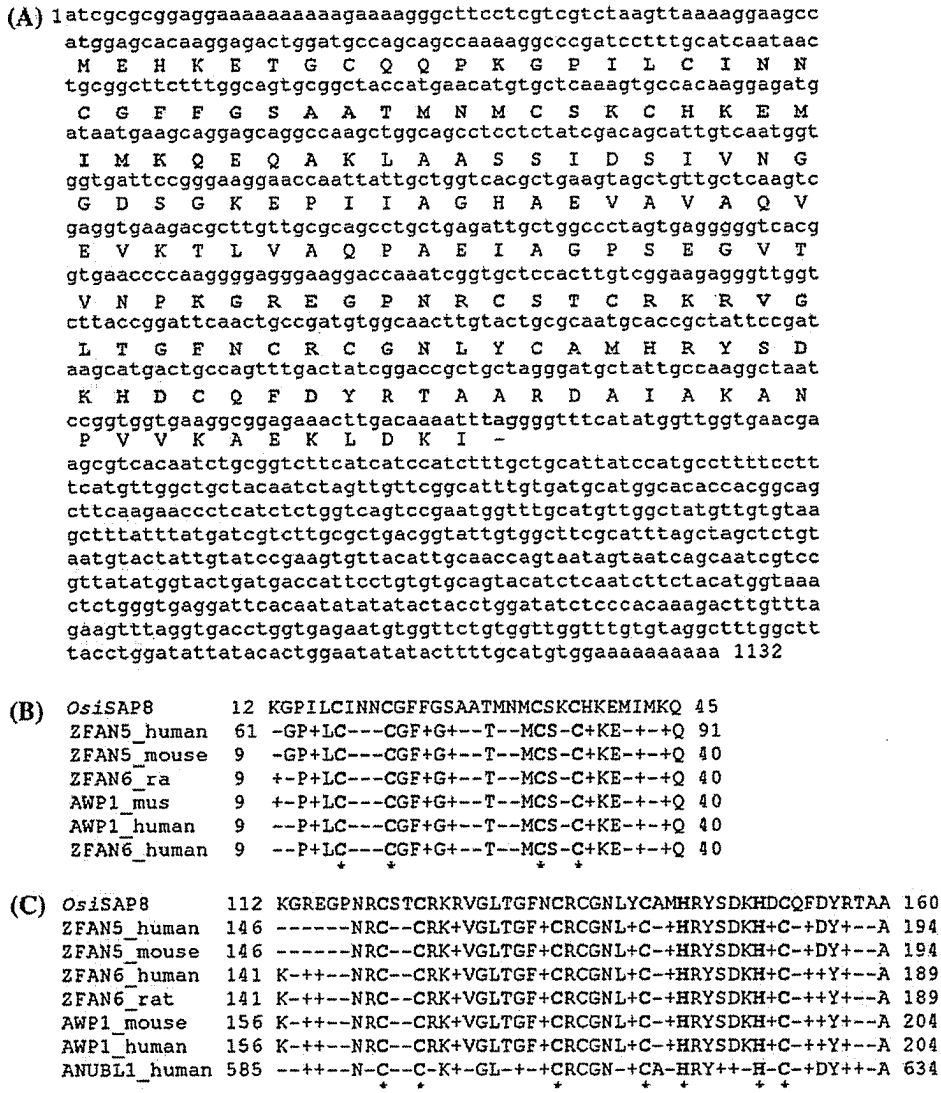

Fig. 1 The nucleotide (1,132 bp) and deduced aminosequence (171 aa) of *OsiSAP8*. The position of start (atg) and stop (tag) codons are indicated in blue letters. The A20 domain and AN1 domain of the peptide are indicated in green and red letters, respectively (**A**). Comparison of deduced aminoacid sequence from *OsiSAP8* and other zinc-finger proteins. Conserved cysteines and histidines are indicated in bold type. Conservation of the aminoacids at N-terminal A20 type zinc-finger (**B**) and the C-terminal AN1 type zinc-finger (**C**) of OsiSAP8 vis-à-vis ZFAN5 (ZnF216) of humans (AAC61801.1) and mouse (AAC42600.1), ZFAN6 of human (AAH05283) and rat (AAH76394), AWP1 (PRK-1 associated protein) protein of mouse (CAC14886.1) and humans (CAC14876.1) and ANUBL1 (AN1, Ubiquitin like protein, AAH48967.1) of humans. Conserved positions of cysteine and histidine residues were marked by asterisk (*)

AN1-type zinc finger domain at the C terminus spanning from 109–152 amino acid region with the consensus sequence of $Cx_2Cx_{9-12}Cx_{1-2}Cx_4Cx_2Hx_5HxC$, where x represents any aminoacid (Fig. 1A). The aminoacid sequence also shows the presence of potential protein kinase phosphorylation site at 63–65 aa region and an N' myristoylation site at 129–134 position. The protein has no detected signal sequence and the pSORT analysis shows that the protein is hydrophilic. Similar domain architecture is found in the human ZNF216 protein involved in immune responses (Huang et al. 2004). In plants, 18 genes from rice and 14 genes from *Arabidopsis* were identified to code for SAP related proteins. SAP gene family members are characterized by the presence of A20/AN1 domains in their putative encoded proteins. Abstract proteins with A20/AN1 zinc finger domains are present in all eukaryotes and are well characterized in animals. In consistent with the finding from animal systems that A20 and AN1 zinc finger domains are usually found associated with each other (Evans et al. 2004), the majority of plant SAP gene family members have both the A20 (present at the N-terminus) and AN1 (present at the C-terminus) zinc finger domains. The A20 zinc-finger domain was first identified in the C-terminus part of a TNFα-inducible protein in human endothelial cells (Dixit et al. 1990; Opipari et al. 1990) and is characterized by multiple Cys2/Cys2 finger motifs (Opipari et al. 1990). This protein plays an important role in regulating the immune response by inhibition of NFκB activity. The AN1 domain was first identified as a putative zinc-finger domain in the proteins coded by the *Xenopus laevis* animal hemisphere-1 (AN1) maternal RNA (Linnen et al. 1993).

Genes showing homology to the conserved region encoding the zinc finger of *OsiSAP8* have been annotated in other plant species such as Maize (AAS00453.1), *Citrus* (ABL67658.1), *Camellia* (ABI31653.1), *Medicago* (ABN08135.1), *prunus* (AAD38146.1) and *Phaseolus* (AA33773). AN1 domain of OsiSAP8 showed 89, 81, 88, 77 and 80% identity and A20 domain showed 96, 87, 72, 72 and 78% identity to its homologues from Maize, *Citrus*, *Camellia*, *Medicago* and *prunus*, respectively. AN1 domain of OsiSAP8 also showed 73% similarity to PVPR3 (*Phaseolus vulgaris* pathogen related protein, Sharma et al. 1992). Apart from the plant homologues, the A20 and AN1 domains also showed homology to several mammalian zinc-finger proteins including the human and mouse PRK1-associated protein AWP1 (CAC14886.1, CAC14876.1, Duan et al. 2000), human and mouse ZFAN5 (AAC61801.1, AAC42600.1 ZNF216 proteins, Scott et al. 1998) and human and rat ZFAN6 proteins (AAH05283, AAH76394, Strausberg et al. 2002). AN1 domain of OsiSAP8 is also homologous to the AN1 domain of human ANUBL1, AN1 and ubiquitin like protein (AAH48967.1, Strausberg et al. 2002). The cysteine and histidines residues of A20 and AN1 that were predicted to be involved in forming the zinc finger were shown to be conserved between the OsiSAP8 and its mammalian homologues (Fig. 1B, C).

OsiSAP8 is a single copy gene as determined by southern analysis (data not shown). Even the Blast analysis revealed that the *OsiSAP8* cDNA sequence showed 99% identity to the genomic sequences of a single contig Ctg020246 from chromosome 6 (Acc. No: AAAA02020246.1) of *indica* rice representing essentially the same gene. To localize *OsiSAP8* gene in the rice genome, its cDNA sequence was used as query for the BLAST search against nipponbare (*Oryza sativa* L, *japonica*) database at GRAMENE (http://www.gramene.org/db/searches/blast). Only a single clone AP003626 located at the locus LOC_Os06g41010 was predicted to be 100% identical to the *OsiSAP8* sequence. According to the physical map of Nipponbare, *OsiSAP8* locates in the region 24.490982–24.493135 MB on the lower arm of chromosome 6. Comparison of full-length cDNA to its genomic DNA sequence revealed that a 2,159 bp genomic DNA sequence, spanning all the exons, represents *OsiSAP8*. It is composed of three exons of 89, 62 and 1,088 bp separated by two introns of 176 and 739 bp, respectively. Both the introns were found in the 5′UTR region and the coding region is continuous without any intron and is encoded exclusively by exon 3. *In silico* analysis also predicted three other splice variants for this gene with different transcript sizes of 1,225, 1,140 and 1,322 bp, because of different splice sites in the 5′UTR. All the splice variants were shown to code for same polypeptide and differ only in the 5′UTR region.

OsiSAP8 is a multiple stress inducible gene

All the SAP gene family members were shown to be inducible by one or the other kind of stress (Vij and Tyagi 2006). *Osi*SAP1 was shown to be induced within 15 min with salt, drought, cold, desiccation, submergence, cold, wounding, ABA and heavy metals (Mukhopadhyay et al. 2004). In silico analysis of 1.5 kb genomic sequence upstream to the transcriptional start site of *OsiSAP8* (retrieved from the sequence of contig of *indica* rice showing 100% identity to *OsiSAP8* cDNA) using PLACE database predicted the presence of several *cis*-acting regulatory elements involved in stress-responsive gene expression such as ABA-responsive element (Simpson et al. 2003), MYC elements (Abe et al. 2003), W-box (Yamamoto et al. 2004) and gibberellic acid responsive element (Morita et al. 1998). Therefore, in this study, an attempt was made to validate the functionality of responsive elements experimentally. For this, northern blot was performed with the total RNA extracted at different time intervals from 8-day-old rice seedlings subjected to various stress treatments, using the *OsiSAP8* cDNA as probe. The gene is induced under several abiotic stresses. The transcript levels increased to higher level within 2 h after salt stress to seedlings and the level continued to increase till 12 h and declined thereafter. However, even at 24 h the mRNA level was more than that of control (Fig. 2A). In case of drought stress, transcript levels peaked within 2 h and the level continued till 24 h (Fig. 2B). With desiccation stress the transcript levels increased to higher level within 2 h and declined after 12 h (Fig. 2C). The transcript levels were increased gradually with the increase in time of exposure of seedlings to heat stress (Fig. 2D). In case of submergence stress, different induction kinetics was observed. The gene was induced within 2 h and maintained higher transcript levels till 24 h, however with a slight decline at 6 h (Fig. 2E). Similar results were observed with *Osi*SAP1 (Mukhopadyay et al. 2004). The transcript level was increased to very high levels within 2 h of wounding stress and was maintained till 4 h, after which its mRNA levels drastically decreased to the level of control at 6 h (Fig. 2F). *OsiSAP8* transcript levels was increased to higher level within 2 h after cold stress to seedlings and the level remained same till 6 h and started declining thereafter. However, even at 12 and 24 h the transcript level was more than that of control (Fig. 2G). The cold induced membrane rigidification is considered to be the primary event in the cold perception by plants (Örvar et al. 2000). The treatment of seedlings with membrane rigidifier DMSO significantly increases the *OsiSAP8* expression (Fig. 2H). The gene was also found to be responsive to different heavy metals. Essentially, treatment with zinc, copper, cadmium and calcium salts led to significant

increase in transcript level by 3 h and continued till 6 h. Manganese and Lithium salts had only marginal effect on mRNA level. Mercury salt reduced the mRNA level possibly because of the toxic effect of mercuric chloride (Fig. 2I). *OsiSAP8* gene responded to ABA at concentrations as low as 1 μM; the expression peaked at 4 h after treatment of seedlings with 1 μM ABA and declined thereafter. However, with the increasing concentration of ABA, the steady-state transcript level was maintained for a longer duration as the mRNA level declined at 12 h with 10 μM ABA and at 24 h with 100 μM ABA (Fig. 2J). This shows that *OsiSAP8* is regulated by ABA in dose dependent manner. Semi-quantitative RT-PCR analysis performed with *OsiSAP8* specific primers indicated that the transcript levels increased within 30 min after the seedlings were subjected to various stresses except the heat stress (Fig. 2K).

This kind of upregulation of *OsiSAP8* within 30 min upon exposure to various stresses like salinity, drought, desiccation, cold, submergence, wounding, heavy metals and ABA suggests that its product might be required during early phase of stress response. Such early induction of gene

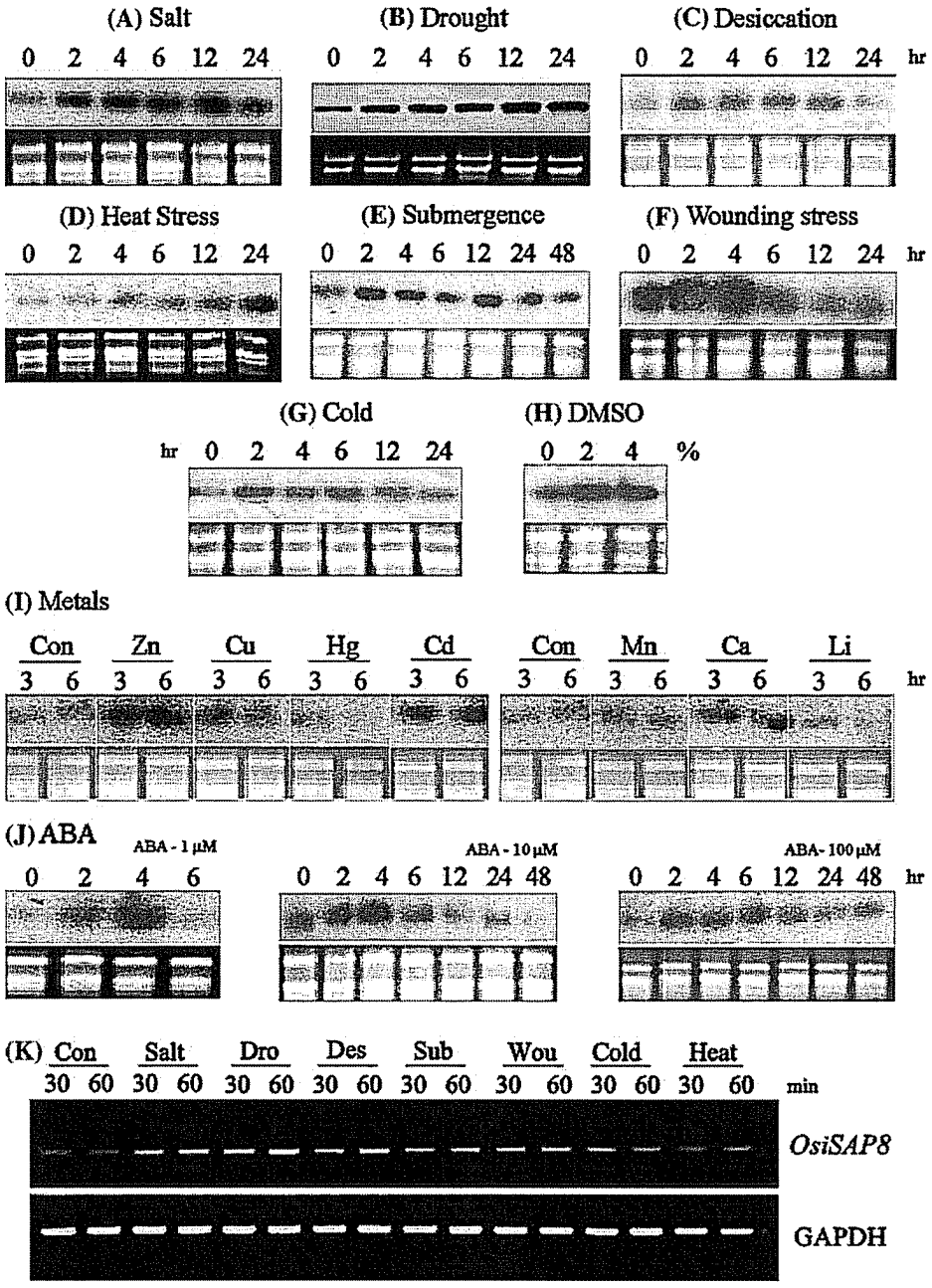

Fig. 2 Expression pattern of *OsiSAP8* after different stresses to rice seedlings: salt (**A**), drought (**B**), desiccation (**C**), heat (**D**), submergence (**E**), wounding (**F**), cold (**G**), DMSO (chemical modulator of membrane fluidity) (**H**), heavy metals (**I**) and ABA (**J**) Con represents the RNA from the control plant with out any stress treatment. 0, 2, 3, 4, 6, 12, 24 and 48 represents the time in hours for which stress treatment was given. *OsiSAP8* cDNA was used as a radiolabeled probe for northern hybridization in all cases. The lower panels in all cases show ethidium bromide-stained rRNA for equivalent loading and RNA quality. RT-PCR analysis was performed with *OsiSAP8* specific primers from the RNA isolated from the rice seedlings subjected to water (Con), Salt, Drought (Dro), Desiccation (Des), Submergence (Sub), Wounding (Wou), Cold and Heat stress (**I**, upper panel) GAPDH amplification was used as internal control (**I**, lower panel) 30 and 60 represents the time in min for which the treatment was given

expression has also been observed under different stress conditions for various genes like desiccation and salt induction of *RD29* (Yamaguchi-shinozaki and Shinozaki 1993, 1994), desiccation induction of *COR47* and *ERD10* (Welin et al. 1994; Kiyosue et al. 1994) and salt induction in case of *OsLEA3* (Moons et al. 1997) as well as desiccation, salt or ABA induction of *RD22* (Abe et al. 1997). The functions of these genes are not well defined. Some genes with known functions like *CDPKs* (Calcium dependent protein kinases, Urao et al. 1994; Saijo et al. 2000) and *CBF* (c-repeat binding factor gene, Shinozaki and Yamaguchi 1997; Kizis et al. 2001) are also induced to high levels early after drought/ salt and cols stress, respectively.

Transgenic tobacco and rice seedlings harboring *OsiSAP8* are tolerant to abiotic stresses

Many stress responsive genes isolated from rice were shown to increase the level of stress tolerance in rice when overexpressed. For example, overexpression of *OsDREB1A*, *OsDREB1B* (Ito et al. 2006) and *OsCDPK7* (Saijo et al. 2000) in rice conferred tolerance to drought, high salt and cold. Similarly, overexpression of *SPL7* (rice heat stress responsive factor, Yamanouchi et al. 2002), *OsCDPK13* (Calcium dependent protein kinase, Abbasi et al. 2004), *OsPTF* (rice Pi starvation induced transcription factor, Yi et al. 2005), *Sub1A* (rice submergence stress tolerant locus, Xu et al. 2006) and *OsNHX4* (rice Na^+/H^+ transporter gene, Fukuda et al. 2004) led to increased levels of tolerance to heat, cold, phosphate deficiency, submergence and salt tolerance.

Overexpression of a gene in both homologous and heterologous system may not always yield same results, for example, overexpression of *Arabidopsis* DREB1a under constitutive promoter led in *Arabidopsis* resulted in improved tolerance to drought, salinity and freezing stress but with severe plant growth retardation under normal conditions of growth (Kasuga et al. 1999). However, constitutive expression of same gene in rice plants improved tolerance to drought and salinity but to a very little extent to cold stress (Oh et al. 2005).

Previously *Osi*SAP1 was overexpressed in tobacco (*Nicotiana tabacum* var. Xanthi) and the transgenic tobacco lines were tolerant to cold (8 ± 1°C for 15 days), dehydration (300–400 mM mannitol) and salt (250 mM NaCl) stresses. It was found that the percentage germination, fresh weight, chlorophyll retention, leaf development of transgenic lines were always better as compared to control (wild-type) plants when grown under stressed conditions (Mukhopadhyay et al. 2004). In this study, we attempted to study the effect of *OsiSAP8* overexpression in both homologous (*Oryza sativa*) and heterologous (*Nicotiana benthamiana*) systems. For this, southern blot analysis of five tobacco lines (TS1, TS2, TS3, TS4 and TS5) and five rice plants (RS1, RS2, RS3, RS4 and RS5) was performed which indicated that the integration pattern of *OsiSAP8* gene was same in TS2 and TS5 lines of tobacco and RS3 and RS4 lines of rice. Thus, four transgenic tobacco lines (TS1, TS2, TS3, TS4) and four rice lines (RS1, RS2, RS3 and RS5) harboring pUbSAP8s, as confirmed by southern blot analysis (data not shown), and constitutively expressing *OsiSAP8* (Fig. 3A, B) were analyzed for stress tolerance in both T_0 and T_1 generations.

Since, *OsiSAP8* was shown to be a multiple stress inducible gene, in this study an attempt was made to test the T_0 transgenic lines for their salinity tolerance by floating leaf-discs on various concentrations of NaCl for 72 h and estimating chlorophyll retention. Similar results were observed with both tobacco and rice transgenic lines. It was observed that the transgenic leaf segments could retain ~95, ~92, ~89 and ~84% of chlorophyll in contrast to the leaf segments from the control plants that could retain only ~78, ~56, ~30 and 24% of chlorophyll when exposed to 200, 400, 600 and 800 mM NaCl, respectively (Fig. 3D, E). Also, the damage caused by stress was reflected in the degree of bleaching in the leaf tissue after 72 h. It was evident that salinity-induced loss of chlorophyll was lower in *OsiSAP8* over expressing lines compared with those from the control plants (Fig. 3C). The results indicated that the transgenic tobacco and rice plants overexpressing *OsiSAP8* have better ability to tolerate salinity stress than control (untransformed) plants. Also *OsiSAP8* transgenic tobacco lines could withstand 800 mM NaCl stress compared to *Osi*SAP1-overexpresing tobacco lines, which could tolerate 250 mM NaCl stress (Mukhopadhyay et al. 2004).

For determining the effect of *OsiSAP8* overexpression on the salt tolerance of T_1 transgenic plants, seeds from both transgenic and control (wild-type) plants of tobacco and rice were grown as described in "Materials and methods". Similar results were observed with both rice and tobacco transgenic lines. Under salt stressed conditions, seeds from transgenic lines showed 100% germination as compared to 20% germination by seeds from control plants after 7 days of germination (Table 1, Fig. 4A, B). Transgenic lines from both tobacco and rice showed better growth under stressed conditions as reflected by the lower levels of reduction in average shoot length, root length and fresh-weight relative to the unstressed control (Table 1, Fig. 4D). It was also observed that the transgenic plants recovered better than control plants, when transferred to normal growth conditions after stress, as evinced by gain in fresh weight (Table 1) and also by visual appearance (Fig. 4C–E). Salt induced chlorosis was observed in both the recovered control and transgenic lines, but was much less in transgenic seedlings (Fig. 4C–E).

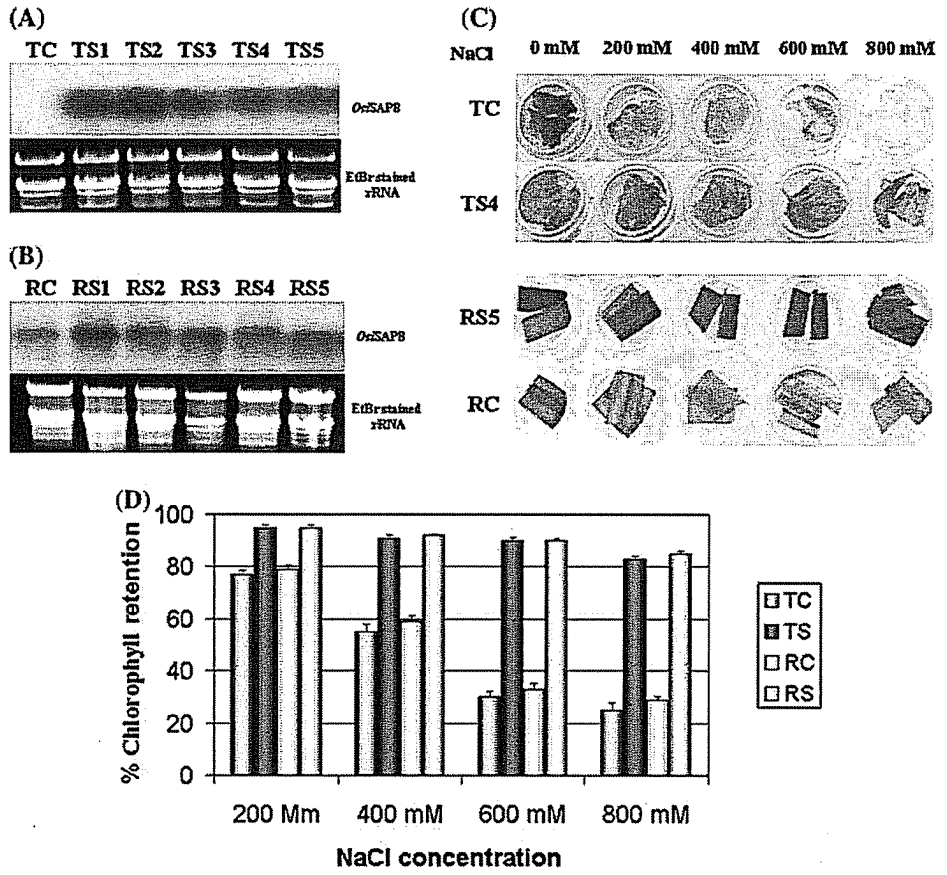

Fig. 3 Analysis of transgenic lines in T_0 generation. Expression of *OsiSAP8* in leaves of untransformed control (TC), transformed (TS1, TS2, TS3, TS4 and TS5) tobacco (**A**) and in untransformed control (RC), transgenic lines (RS1, RS2, RS3 and RS5) if rice (**B**). *OsiSAP8* cDNA was used as a radiolabeled probe for northern hybridization in all cases. The lower panels in all cases show ethidium bromide-stained rRNA for equivalent loading and RNA quality. Leaf disc assay was performed to analyze the salt tolerance levels of T_0 transgenic plants by floating the leaf segments from control and transgenic plants on 0 mM (control), 200, 400, 600 and 800 mM NaCl solution. The phenotypic appearance of the transgenic leaf segments (TS4 and RS5) as compared to wild-type (control, TC, RC) leaves was observed (**C**). Similar kind of results was obtained from all the transgenic plants. At the end of 72 h of salt stress, % chlorophyll retention in the leaf discs was measured. The error bars in the graph represent the standard deviation of the values taken from all the five transgenic lines of both tobacco and rice (**D**)

Evaluation of *OsiSAP8*-overexpressing lines for dehydration stress tolerance revealed that the percentage germination of wild-type seeds from both tobacco and rice was much less compared with transgenic seeds over a period of 7 days. Although 95% germination was observed in transgenic lines under both 300 mM (Fig. 5A, C; Table 1) and 400 mM (Fig. 5B, D; Table 1) mannitol stress, the growth (in terms of fresh-weight, root length and shoot length) was better when grown under 300 mM mannitol stress condition as compared to 400 mM mannitol stress (Table 1, Fig. 5E, F). However, the fresh weight, shoot length and root length of transgenic lines, under both conditions of stress, was much higher compared with that of wild-type as reflected on quantitative estimation (Table 1) and visual appearance (Fig. 5E, F). Significant improvement in the fresh-weight, root length and shoot length gain of seedlings from transgenic rice lines under stress was observed over a recovery period of 8 days. Over a similar period, fresh-weight gain in wild-type seedlings was minimal. Also, it was observed that both control and transgenic seedlings grown under 0.3 M Mannitol stress showed better recovery as compared to seedlings grown under 0.4 M Mannitol stress (Fig. 5G, H). Though, three leaf stage was observed in the transgenic seedlings recovered from both 0.3 and 0.4 M mannitol stress, the elongation of shoot was effected with 0.4 M Mannitol (Fig. 5G, H). The results indicated that though the drought stress retarded the growth of transgenic seedlings by 50%, they fared better than the control seedlings, which showed around 90% growth retardation under stress.

Transgenic lines were also analyzed for cold tolerance in T_1 generation as described in "Materials and methods". In

Table 1 Effect of salt and drought stress on tobacco and rice seedlings from wild-type (control) and *Osi*SAP8 overproducing transgenic lines on percentage germination; percentage decrease in root length, shoot length and fresh weight relative to that of unstressed control plants during germination (7 days of germination under stress conditions) and recovery stage (after 8 days of recovery)

Parameter	Salt (NaCl)				Drought (Mannitol)							
	150 mM		200 mM		300 mM				400 mM			
	TC	TS	RC	RS	TC	TS	RC	RS	TC	TS	RC	RS
Germination												
% Germination	20 (0.50)	100 (0.00)	22.5 (0.24)	96 (0.12)	50 (0.18)	98 (0.50)	40 (0.32)	93 (0.08)	40 (0.04)	86 (0.50)	25 (0.12)	85 (0.18)
% Decrease in shoot length	80 (0.72)	80 (0.18)	70 (0.03)	35 (0.08)	70 (0.32)	36 (0.72)	82 (0.12)	45 (0.32)	85 (0.02)	47 (0.18)	90 (0.24)	50 (0.32)
% Decrease in root length	83 (0.03)	80 (0.04)	72 (0.18)	10 (0.12)	91 (0.04)	38 (0.18)	84 (0.03)	40 (0.24)	92 (0.02)	44 (0.50)	80 (0.12)	45 (0.08)
% Decrease in fresh weight	80 (0.02)	22 (0.08)	60 (0.08)	25 (0.02)	78 (0.50)	26 (0.18)	70 (0.04)	42 (0.12)	83 (0.05)	43 (0.50)	78 (0.04)	50 (0.12)
Recovery												
% Gain in shoot length	10 (0.72)	50 (0.24)	10 (0.18)	50 (0.32)	nd	nd	12 (0.12)	90 (0.04)	nd	nd	10 (0.04)	40 (0.12)
% Gain in root length	19 (0.50)	30 (0.50)	30 (0.03)	60 (0.12)	nd	nd	15 (0.24)	70 (0.32)	nd	nd	10 (0.08)	50 (0.32)
% Gain in fresh weight	8.0 (0.02)	47 (0.04)	20 (0.24)	80 (0.32)	nd	nd	18 (0.12)	85 (0.18)	nd	nd	10 (0.02)	63 (0.24)

Values indicate the average of observations made from two independent experiments, each performed with five samples of wild-type (TC—Tobacco control; RC—Rice control) and five samples from each of four transgenic lines (TS: TS1–TS4 tobacco transgenic lines and RS: RS1–RS3, RS5 rice transgenic lines) per experiment. Variance of the two experiments was indicated in the brackets

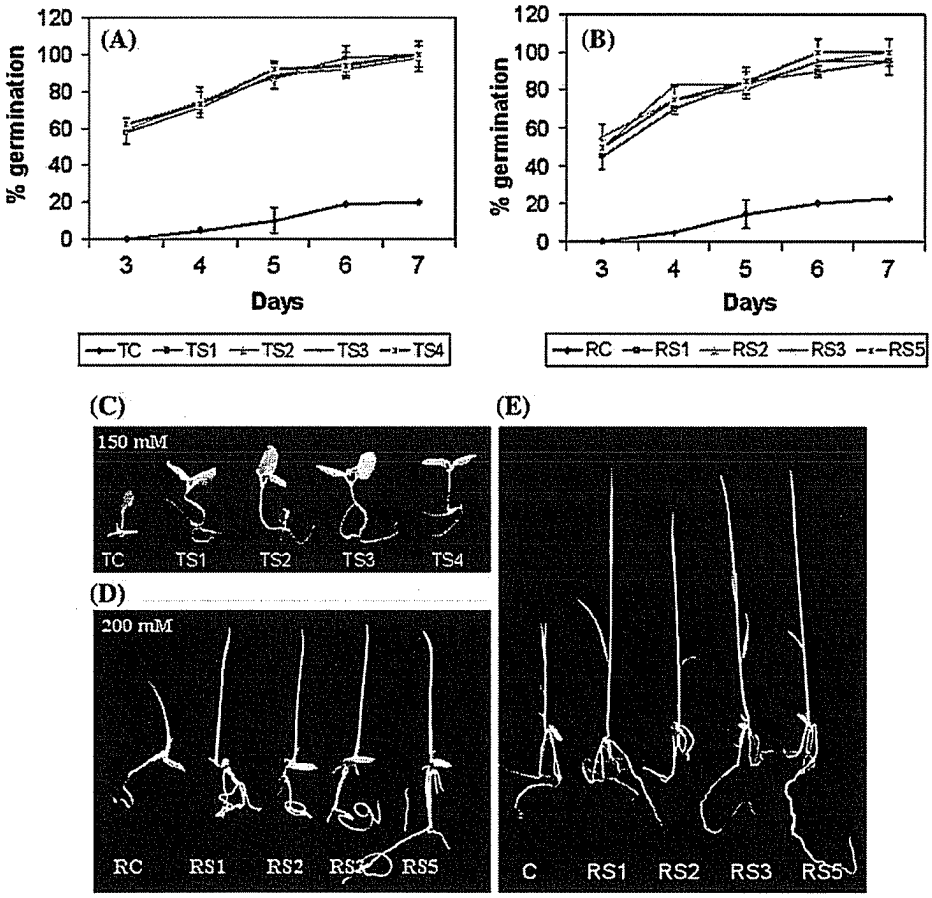

Fig. 4 Effect of salt stress on seedlings from wild-type and T_1 progenies of tobacco (TS1, TS2, TS3 and TS4) and rice (RS1, RS2, RS3 and RS5) transgenic lines overexpressing *OsiSAP8*. The seeds were allowed to germinate in presence of NaCl (150 mM for tobacco and 200 mM for rice) and germination percentage was calculated at the end of 7 days (**A**, **B**). Photograph of the representative seedlings of tobacco control (TC) and four transgenic lines (TS1, TS2, TS3 and TS4) were taken after 15 days of recovery (**C**). Photographs taken for representative seedling of rice control (RC) and transgenic lines (RS1, RS2, RS3 and RS5) after 8 days of germination on salt (**D**) and 7 days after recovery (**E**) are also represented

case of tobacco seedlings, no significant differences in the appearance was noticed after exposure to 4 ± 1°C for 4 days. However, under similar conditions, leaves of the wild-type rice seedlings rolled in and dried while the leaves from transgenic seedlings remained fresh (Fig. 6B). The transgenic lines from both tobacco and rice recovered well as indicated by the gain in the fresh-weight. Transgenic rice seedlings gained 40% fresh-weight as compared to only 3% gain by control rice seedlings after 7 days of cold recovery. After 15 days of recovery, stressed tobacco transgenic seedlings gained 76–82% fresh weight as compared to 20% by control seedlings grown under similar conditions. Phenotypically, the transgenic seedlings appeared better than the control seedlings with the appearance of third and fourth leaves with an elongated internode. Whereas in case of control tobacco seedlings only the first two leaves were prominent with a small third leaf (Fig. 6A). *Osi*SAP1-overexpressing tobacco seedlings were shown to withstand 8 ± 1°C for 15 days (Mukhopadhyay et al. 2004). Based on these observations, it may be argued that overexpression of *OsiSAP8* both in homologous and heterologous systems conferred tolerance to high salt, drought and cold stresses equally.

Productivity of transgenic rice plants was not affected under salt and drought stress

It was observed that the overexpression of *OsiSAP8* led to ~50% yield penalty in transgenic lines under normal conditions of growth (Table 2). So in another set of experiments, an attempt was made to know the effect of salt (NaCl) treatment and water-deficit on productivity of transgenic rice lines overexpressing *OsiSAP8*. Stress treatments were given to the mature plants as described in "Materials and methods". The control plants were severely affected in both the cases and eventually died within 25 days. However, transgenic plants did not show any sign of stress, continued to grow, reached maturity, flowered and set seeds (Fig. 5I). In case of salt stress, the flowering time was delayed in by a week but no change in the flower morphology or the number of panicles and flowers was observed as compared to unstressed transgenic lines. It was observed that the yield decreased significantly by ~49–55% compared to that of unstressed control plant (Table 2). However, no penalty was observed when compared to unstressed transgenic lines (Table 2). Hence, the productivity was not affected in transgenic rice lines under

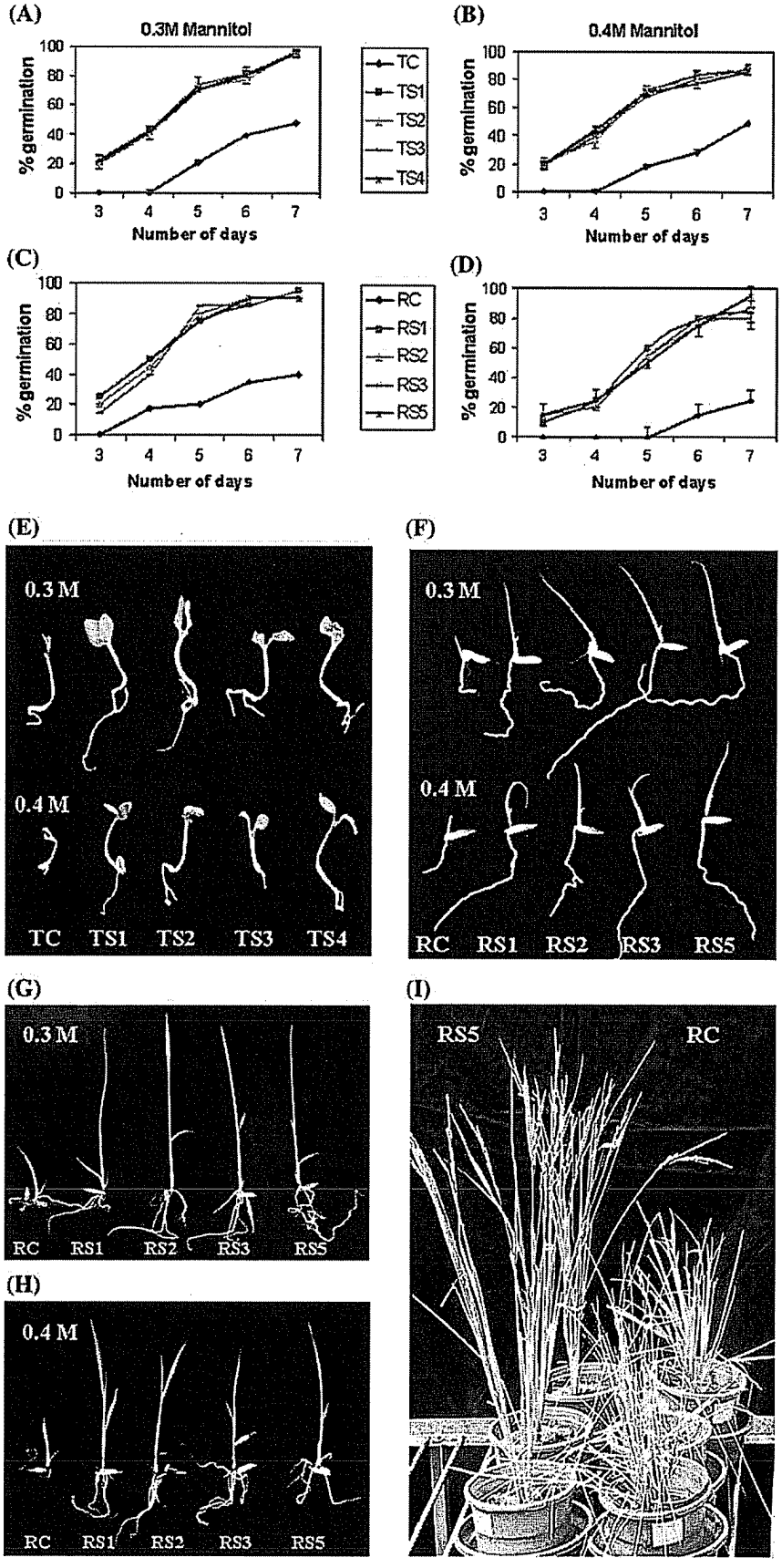

Fig. 5 Effect of dehydration stress on seedlings from control and *OsiSAP8*-overexpressing transgenic lines of rice and tobacco. Seeds were germinated on 0.3 M (**A**, **C**) and 0.4 M mannitol (**B**, **D**) and percentage germination was calculated. Standard deviation of the values taken for five samples is shown in **A**, **B**, **C** and **D**. Photograph of representative seedlings of control (RC—rice control, TC—tobacco control) and four transgenic lines (Tobacco—TS1, TS2, TS3 and TS4; Rice—RS1, RS2, RS3 and RS5) taken after 7 days of germination on 0.3 M mannitol (**E**, **F** upper panel) and 0.4 M mannitol (**E**, **F** lower panel) is given. After 8 days of recovery, rice seedlings grown on 0.3 M mannitol (**G**) recovered better than those grown on 0.4 M mannitol (**H**). The rice transgenic lines did not show any symptom of drought and grew well under water deficit conditions for 23 days in contrast to the control plants that wilted and died (**I**)

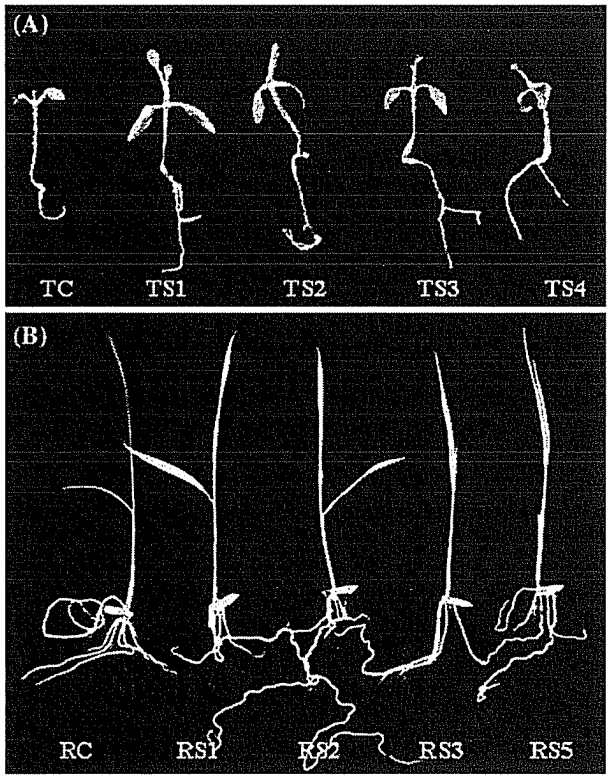

Fig. 6 Effect of cold stress on seedlings from control (wild-type) and *OsiSAP8* overexpressing transgenic lines of rice and tobacco. Twelve-day-old seedlings from tobacco and rice were grown at 4 ± 1°C for 4 days and transferred to culture room conditions for recovery. After 15 days of recovery, the transgenic tobacco lines (TS1, TS2, TS3 and TS4) recovered better and formation of new leaves with elongated internode was observed (**A**). After 4 days of cold stress, the leaves of transgenic rice lines (RS1, RS2, RS3 and RS4) looked fresh compared to the leaves of control rice seedling (RC) that rolled inside and dried (**B**)

drought and salt stresses compared to unstressed transgenic lines.

Constitutively active promoters are not always desirable for plant genetic engineering because overexpression of a transgene may compete for energy and building blocks for synthesis of proteins or RNA that are also required for plant growth under normal conditions (Kathuria et al. 2007). Several inducible and tissue/stage specific promoters are available and have been studied in rice, which provides a way to express the transgene very specifically in the target plant. Expression of genes for stress tolerance could be engineered better by deploying stress-inducible promoters or their elements. Many such promoters were studied in rice (summarized in Kathuria et al. 2007). Expression of *OsiSAP8* under stress inducible promoters in future would give better insight into the effect of *OsiSAP8* overexpression during the stress conditions.

Table 2 Effect of drought (water deficit for 23 days during anthesis) and salt stress (200 mM NaCl) on the phenotypes of rice T_1 transgenic plants

Phenotype	Unstressed wild-type (control)	Unstressed transgenic rice	Water deficit					200 mM NaCl				
			C	RS1	RS2	RS3	RS5	C	RS1	RS2	RS3	RS5
Height of the plant (cm)	91 (0.50)	80 (0.50)	–	66 (0.18)	71 (0.24)	75 (0.02)	77 (0.50)	–	76 (0.32)	70 (0.12)	74 (0.04)	69 (0.12)
Length of the leaf (cm)	52 (0.12)	50 (0.32)	–	50 (0.08)	46 (0.50)	49 (0.12)	50 (0.32)	–	45 (0.50)	47 (0.08)	50 (0.12)	52 (0.04)
Chlorophyll (mg/g FW)	4.2 (0.00)	4.0 (0.00)	–	4.0 (0.00)	4.1 (0.00)	3.9 (0.00)	4.2 (0.00)	–	4.1 (0.00)	4.0 (0.00)	3.8 (0.00)	4.0 (0.00)
No. of spikes per plant	8 (0.12)	13 (0.04)	–	8 (0.02)	9 (0.08)	8 (0.04)	10 (0.02)	–	11 (0.04)	12 (0.02)	13 (0.00)	11 (0.00)
Length of the spike (cm)	20 (0.72)	14 (0.03)	–	13 (0.02)	12 (0.02)	11 (0.08)	13 (0.18)	–	14 (0.00)	15 (0.32)	13 (0.02)	13 (0.04)
No. of flowers per spike	84 (0.02)	70 (0.00)	–	60 (0.00)	59 (0.08)	61 (0.18)	64 (0.32)	–	63 (0.72)	65 (0.04)	69 (0.08)	61 (0.00)
Number of mature seeds per plant	510 (1.80)	240 (2.20)	–	222 (0.72)	254 (0.98)	203 (0.50)	231 (0.50)	–	225 (0.08)	256 (2.64)	233 (1.44)	259 (0.18)
Yield penalty compared to unstressed control (%)	–	52.94	–	56.4	50.196	60.19	54.7	–	55.88	49.8	54.31	49.21

Values are the average of observations made from two separate experiments each involving a sample size of five plants. Values in the brackets represent the variance of the two experiments

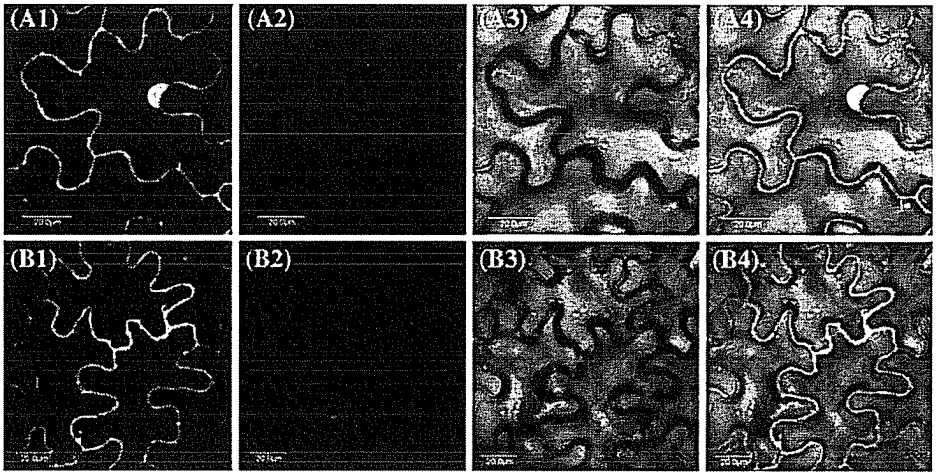

Fig. 7 Subcellular localization of GFP alone and GFP-SAP8 fusion protein during transient expression in *N. benthamiana* leaves. The fluorescence was visualized using confocal microscopy. GFP fluorescence (green channel), red channel (autofluorescence of plant tissue), bright field channel and channel overlays of green, red and bright field channel are shown. Free GFP was expressed both in the cytoplasm and nuclei (**A1–A4**) whereas GFP-SAP8 fusion protein was localized only in the cytoplasm (**B1–B4**)

OsiSAP8 is a cytoplasmic protein and might carry out its functions via protein–protein interactions aided by its A20 and AN1 zinc finger domains

The exact role of *OsiSAP8* in stress tolerance is not clearly understood. It is speculated that rice SAP gene family members would probably fall into the category of regulatory genes induced during stress response since they code for zinc-finger containing domain. Generally many of the zinc finger domain proteins act as transcriptional factors. However, it was hypothesized that OsiSAP1, may not act as transcriptional factor since it lacks any nuclear localization signal and DNA binding domain. It was also suggested that OsiSAP1 might carry out its function via protein–protein interactions (Mukhopadhyay et al. 2004). SAP proteins have homology to human proteins A20 and ZNF216 that negatively regulates the NF-κB activation pathway (Cooper et al. 1996; Evans et al. 2004). ZnF216 has the A20 zinc-finger at the N-terminus and the AN1 zinc-finger at the C-terminus. ZNF216 interacts with components of the immune response like RIP, IKKγ and TRAF6 mediated by the A20 zinc-finger domain and the AN1 zinc-finger domain, showing that both the zinc-finger domains are involved in regulating the immune response. Also co-immunoprecipitation experiments showed that the ZnF-A20 and ZnF-AN1 domains of ZNF216 could interact with each other, whereas ZNF216 could form homo-oligomers or hetero-oligomers with A20 (Huang et al. 2004).

In silico analysis of *OsiSAP8* gene product did not predict any potential nuclear localization signal or DNA binding domain. Instead Sosui analysis and pSORT analysis indicated it to be a hydrophilic soluble cytoplasmic protein. In this study, we performed transient expression of green fluorescent protein (GFP) fused with *OsiSAP8* coding region, in the epidermal cells of *N. benthamiana*. When expressed alone, GFP was distributed uniformly in the cells (Fig. 7A1–A4). GFP-SAP8 fusion protein was

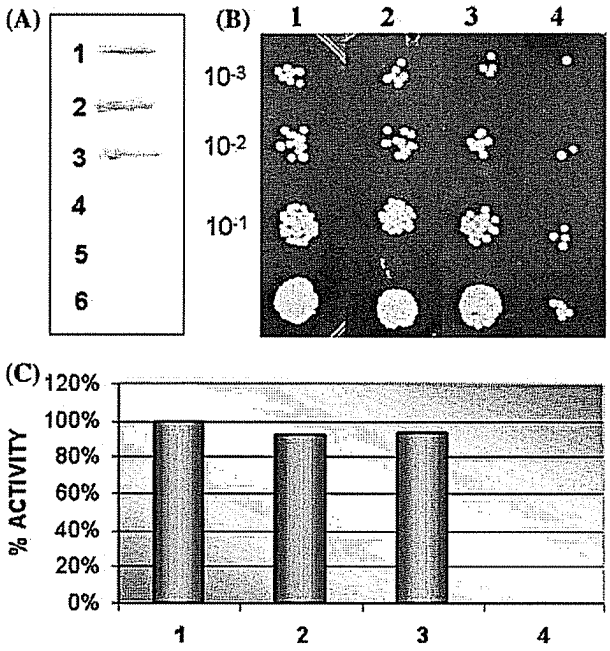

Fig. 8 Yeast two hybrid analysis. EGF 48+ yeast cells were co-transformed with pEGA20–pJGA20 (1), pEGA20–pJGAN1 (2), pEGAN1–pJGA20 (3), pEGAN1–pJGAN1 (4), pEG202–pJGAN1 (5) and pEG202–pJGA20 (6) and the interactions of A20 and AN1 domains were studied by β-galactosidase assay (**A**), leucine prototrophy assay (**B**) and Quantitative ONPG assay (**C**). The host cells harboring either pJGA20 or pJGAN1 along with pEG202 empty vector served as negative control. For leucine prototrophy assay the co-transformants grown on liquid culture lacking tryptophan and histidine were serially diluted and 10 µl of each dilution till 10^{-3} was plated on selective medium lacking tryptophan, histidine, uracil and leucine. The % activity of β-galactosidase represented as bars relative to each other (**C**). The interaction with higher activity was taken as 100%

specifically localized to cell cytoplasm (Fig. 7B1–B4), indicating that unlike many zincfinger containing proteins, OsiSAP8 is a cytoplasmic protein. Thus, as in the case of ZnF216, *OsiSAP8* gene product may carry out its functions

Table 3 Quantitative assay for β-galactosidase

Standard deviations are shown based on three independent experiments. β-galactosidase activity is expressed as percentage of maximal enzymatic activity taking the stronger interaction as 100%

Number	Construct	OD_{420} (SD)	β-gal units	% of maximal
1	pEGA20–pJGA20	0.186 (0.004)	9.54	100
2	pEGAN1–pJGA20	0.170 (0.002)	8.71	91.2
3	pEGA20–pJGAN1	0.172 (0.002)	8.80	92.2
4	pEGAN1–pJGAN1	–	–	–
6	pEG202–pJGAN1	–	–	–
7	pEG202–pJGA20	–	–	–

via protein–protein interactions aided by A20 and AN1 domains. To investigate the possible interactions between A20 and AN1 domains of OsiSAP8, a Yeast two-hybrid analysis was performed using the A20 and AN1 zinc-finger domains of OsiSAP8 fused to AD (activation domain) and BD (DNA binding domain) domains of pJG4-5 and pEG202 yeast vectors. Qualitative β-galactosidase assay revealed that yeast cells co-transformed with pJGA20–pEGA20; pJGA20–pEGAN1 and pJGAN1–pEGA20 showed blue staining reflecting a specific A20–A20 and A20–AN1 interaction. No blue staining was seen with pJGAN1–pEGAN1 interaction (Fig. 8A) Yeast cells transformed with pJGAN1 and pEG202 empty vector, pJGA20 and pEG202 empty vector, that served as negative control also remained white showing no interaction. In addition, leucine prototrophy assay also showed that only the cells showing A20–A20 and A20–AN1 interactions led to growth of cells on a leucine-deficient dropout medium (Fig. 8B). The yeast cells co transformed with pEGAN1–pJGAN1; pJGAN1–pEG202 and pJGA20–pEG202 did not show any growth on leucine dropout medium (data not shown). Therefore it can be suggested that A20 interacts with itself and also with AN1 domain and AN1 does not interact with itself. The qualitative β-galactosidase assay revealed that A20–A20 interaction is stronger than the A20–AN1 interaction (Fig. 8C, Table 3). Hence, it may be suggested that ZnFA20 domain may help in the formation of homodimers or heterodimers to carry out the functions of the OsiSAP8. These results along with the early nature of its induction upon stress treatments indicate that *OsiSAP8* gene product might act in the early phase of signal transduction pathway of stress response.

The present study provides the preliminary evidence that that the A20 and AN1 domains interact in similar fashion as in animal proteins and hence clues for the possible role of SAP family members could be obtained from studies done with human A20 protein. A20 protein from animal systems was shown to downregulate NF-κB by targeting proteins involved in its signaling for degradation through the Ubiquitin proteasome pathway (Wertz et al. 2004; Lee et al. 2006; Penengo et al. 2006). So, SAP gene family members, as in the case of animal systems may be involved in downregulating the pathway associated with abiotic stress injuries such as cell death by ubiquitinylating the key proteins and hence targeting them to degradation (Vij and Tyagi 2006).

In conclusion, in this study, *OsiSAP8*, a member of SAP gene family from rice was isolated. It is found to be coding for a cytoplasmic zinc finger protein that might act early in the signal transduction of various stress responses. Its overexpression in both homologous (rice) and heterologous (tobacco) system lead to an increase in stress tolerance, as determined by salt-, drought-, and cold-tolerance assays. It was found that the chlorophyll retention, percentage germination, fresh weight, root and shoot elongation and leaf development were much better in both rice and tobacco transgenic lines, as compared to control plants, under stress and recovery conditions. However, the transgenic plants showed a yield penalty of 50% under unstressed conditions and the same yield was maintained under salt and drought stresses during anthesis, which is much better compared to no seed set in control stressed plants. Future experiments with *OsiSAP8* under stress inducible promoters might help overcome the problem of yield penalty. The role of *OsiSAP8* in other stresses that have been shown to induce its expression still need to be determined.

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